Expression, Purification, and Characterization of Isoform 1 of the Plasma Membrane Ca^{2+} Pump

FOCUS ON CALPAIN SENSITIVITY*

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The plasma membrane Ca^{2+} ATPase isoform 1(PMCA1) is ubiquitously distributed in tissues and cells, but only scarce information is available on its properties. The isoform was overexpressed in Sf9 cells, purified on calmodulin columns, and characterized functionally. The level of expression was very low, but sufficient amounts of the protein could be isolated for biochemical characterization. The affinity of PMCA1 for calmodulin was similar to that of PMCA4, the other ubiquitous PMCA isoform. The affinity of PMCA1 for ATP, evaluated by the formation of the phosphorylated intermediate, was higher than that of the PMCA4 pump. The recombinant PMCA1 pump was a much better substrate for the cAMP-dependent protein kinase than the PMCA2 and PMCA4 isoforms. Pulse and chase experiments on Sf9 cells overexpressing the PMCA pumps showed that PMCA1 was much less stable than the PMCA4 and PMCA2 isoforms, *i.e.* PMCA1 had a much higher sensitivity to degradation by calpain. The effect of calpain was not the result of a general higher susceptibility of the PMCA1 to proteolytic degradation, because the pattern of degradation by trypsin was the same in the three isoforms.

The plasma membrane Ca^{2+} ATPase (PMCA)[1] pumps Ca^{2+} out of the cell, reducing its concentration in the cytosol to the level compatible with the messenger function (1). In excitable tissues, *e.g.* heart, it does so in concert with the Na$^+$/Ca^{2+} exchanger (2). The pump has been detected in all mammalian cells studied so far (1, 3), although differences in the level of its expression have been observed (4). cDNA cloning has identified four independent PMCA transcripts in human and rat tissues, and the corresponding human genes have been located on four different chromosomes (3, 5–7). The primary transcripts are spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms (1). The cloning work has indicated that certain transcripts of the PMCA isoforms and of their splicing products have striking tissue-specific expression (8–14). For example, although PMCA1 and PMCA4 have been detected in all tissues examined, PMCA2 and PMCA3 have only been found in significant amounts in the brain and a few other tissues. These results on the transcripts have been confirmed at the protein level with isoform-specific antibodies (13). Quantitative analysis has shown that, in general, the human PMCA1 product is more abundant than the PMCA4, both at the mRNA (4) and the protein level (13).

The overexpression of the PMCA4 and the PMCA2 proteins has permitted the study of their biochemical properties (15–19). A number of methods (transient transfection, stable cell lines, vaccinia virus as a vector) have been used (20–22), significantly advancing knowledge on the differential properties of the isoforms. Strikingly, however, very little information is available on PMCA1, which is the most common isoform. Only one successful attempt to express this isoform (a truncated variant C) has so far been reported (23). Difficulties in expressing this isoform have been repeatedly reported (23, 24).

The PMCA pump is a substrate of intracellular proteases. Initial observations had indicated that it was a substrate for the Ca^{2+}-dependent protease calpain (CANP, EC 3.4.22.17) (25), and more recent work has shown that effector caspases (*e.g.* caspases 1 and 3) also cleave PMCA pump isoforms (22, 26). Both activation and inactivation of PMCA2 and PMCA4 by caspases have been reported (22, 26). Calpain attacks calmodulin binding enzymes (27), removing portions of the calmodulin binding sequence and leading to their "irreversible" activation. The protease contains regions with strong homology to calmodulin (domains IV and VI; Refs. 28, 29), which may be important in directing it to its target sequences. Support for this concept was provided by the binding of calpain to the calmodulin binding domain of the erythrocyte pump (27). The proteolysis of the pump by calpain removes most of the C-terminal portion protruding into the cytosol, leaving behind a constitutively active fragment of about 124 kDa. The process has been studied on erythrocyte membranes or on the pump purified from them, *i.e.* on a mixture of the pumps isoforms present in erythrocytes, PMCA1, and PMCA4 (13). Because PMCA4 represents at least 80% of the total erythrocyte pump, it was not surprising that most of the peptides isolated after calpain digestion should have derived from this isoform (30). Nevertheless, the failure to detect high molecular mass peptides deriving from PMCA1 indicated that the latter isoform had different calpain sensitivity.

The work described here was performed to characterize biochemically the human PMCA1 isoform, including its calpain sensitivity. The pump was expressed in Sf9 insect cells with the help of the baculovirus system, but a complex DNA manipula-

tion was required to generate a full-length PMCA1 clone devoid of mutations, which could be transferred to the baculovirus. The protein became expressed at lower levels than the PMCA2 and PMCA4 isoforms but could still be purified on a calmodulin-Sepharose column. The purified PMCA1 was activated by calmodulin, interacting with it with an affinity similar to that of PMCA4. However, it had a distinctly higher affinity for ATP in experiments measuring the formation of the phosphoenzyme intermediate. In agreement with previous observations (31), the purified PMCA1 was a better substrate for the cAMP-dependent kinase than PMCA4 and PMCA2.

The PMCA4, PMCA2, and PMCA1 proteins were tested for calpain sensitivity *in vitro*. Although the PMCA1 isoform was rapidly and completely degraded, the PMCA4 and PMCA2 proteins were proteolyzed much more slowly and only to large active fragments. The digestion patterns of the PMCA2 and PMCA4 isoforms also differed. Whereas only the calmodulin binding domain and the sequence downstream of it were quantitatively removed in the case of PMCA4, a more complex pattern of degradation prevailed in the PMCA2 protein.

EXPERIMENTAL PROCEDURES

Materials—m-CANP was isolated from freshly collected human erythrocytes as described in Ref. 27. The TNM-FH medium was from Sigma. Antibiotics and fetal calf serum were purchased from Invitrogen. The monoclonal antibody 5F10 was obtained from Milan Analytic AG (Hoffmann-La Roche). Nitrocellulose was from Schleicher & Schuell (Dassel, Germany). Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate were from Promega (Madison, WI). All other reagents were of the highest purity grade commercially available.

Construction of the Expression Vectors—To construct a full-length cDNA of human PMCA1, two clones (t6, t8.1) were used (32). The human PMCA1 isoform was used (also known as PMCA1b). The vectors used for the constructions were the pTZ18/19 (United States Biological, Swampscott, MA), the pSG5 (Stratagene, GmbH, Zürich, Switzerland), and the pVL1393 (provided by Dr. M. D. Summers, Texas A&M University, College Station, TX). DNA was purified by CsCl centrifugation (33) or by Qiagen columns (Quiagen, Chatsworth, CA). Different attempts to ligate fragments together to obtain a full-length construct resulted in a number of deletion-containing products. The use of PCR-directed mutagenesis to create suitable restriction sites or cDNA fragments was similarly unsuccessful. In a first step an *Xba*I, located in vector sequences proximal to the 3′-end of the PMCA1 and *Bam*HI (nt 950, HUMPMPCA, GenBank™ accession number J04027) fragment, was subcloned together with a 900-bp *Sal*I-*Bam*HI (nt 950, J04027) fragment; the *Sal*I was located in the vector proximal to the 5′-end, in the pSG5 *Sal*I-*Xba*I cut vector. This vector was used because it yielded the highest amount of DNA of all those that were tried. No mutations were observed using this vector. After modifying the *Xba*I to a *Kpn*I site, the PMCA1 cDNA was cut by *Sal*I, blunt ended, cut by *Kpn*I, and subcloned in pVL1393. The bacteria bearing the pVL1393 PMCA1 construct produced very low amounts of plasmid DNA. Generally, 2–4 μg of DNA could be purified from 250–500 ml of liquid cultures in 2XYT, corresponding to about one plasmid copy per bacterial cell.

Culturing of Sf9 Cells and Construction of a Recombinant Baculovirus—*Spodoptera frugiperda* (Sf9) cells were grown in TNM-FH medium supplemented with 10% fetal calf serum and 50 μg/ml gentamicin (both from Invitrogen) at 30 °C. PMCA1-containing DNA purified by two CsCl gradient centrifugations was cotransfected with Bak-Pak DNA (Invitrogen) to Sf9 cells using the $Ca_3(PO_4)_2$ precipitation method as described (34). The recombinant baculoviruses were identified by visual inspection of the viral plaques after plating on Sf9 cells and analyzed by dot blotting with PMCA1-specific probes. Putative recombinant viral plaques were checked for the correct insertion in the presence of 150 μg/ml X-gal (5-bromo-chloro-3-indolyl-b-D-galactoside; Bachem Feichemikalien AG, Dübendorf, Switzerland). The recombinant plaques were amplified and titrated as described (34).

More than 20 different recombinant viruses were isolated and tested for their ability to express the PMCA1 pump. Seven of them (see also Fig. 3*A*) expressed a protein of about 140 kDa. The band was absent in non-infected Sf9 cells. From some clones, viral DNA was prepared and analyzed by Southern blotting (33). Viral DNA was obtained from sucrose gradient purified viruses (34). As expected, digestion with *Bam*HI (a *Bam*HI site is present in the pVL1393 vector just upstream

of the PMCA1 sequence) and *Pst*I-*Bam*HI produced one fragment of 1000 bp and two fragments of 500 and 400 bp, respectively, which were recognized by probes spanning the coding sequence of PMCA1. Digestion with *Hin*dIII resulted in a PMCA1-specific fragment of 9500 bp, confirming that the PMCA1 was inserted at the right place in the genome of the baculovirus. After transferring the PMCA1 DNA to the latter, problems with the stability of the DNA were no longer encountered.

Preparation of RNA, Northern Blotting, and Preparation of Digoxigenin Probes—2–4 × 10^7 Sf9 cells were infected with recombinant baculovirus for 48 h. The cells were collected, washed twice in 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, and resuspended in 600 μl of 20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ on ice before the addition of 12.5 mM vanadyl-ribosyl complex (Invitrogen). Cytosolic RNA was then purified as described in Ref. 35. Northern blotting was performed essentially as described in Ref. 36. DNA fragments were labeled with digoxigenin-dCTP (Roche Applied Science), using the protocol suggested by the manufacturer.

Preparation of Membranes—Sf9 cells were seeded at a density of 10^4/cm^2 and left to attach for 1 h. They were infected at a multiplicity of infection of 5–10 for 1 h, and then the inoculum was replaced by fresh medium. After 48 h the infected cells were collected, washed twice with TBS (25 mM Tris-HCl, pH 7.5, 150 mM NaCl), and resuspended in 2 ml of 10 mM Tris-HCl, pH 7.5 (1 × 10^7 cells/ml). After 10 min on ice, the cells were homogenized in the presence of 75 μg/ml phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 μg/ml leupeptin, 5 μg/ml antipapain, and 5 μg/ml pepstatin. The homogenate was diluted by adding an equal volume of 10 mM Tris-HCl, pH 7.5, 20% sucrose, and 300 mM KCl and centrifuged at low speed (750 × *g*) for 5 min. The supernatant was centrifuged at 100,000 × *g* and resuspended in 20 mM HEPES-KOH, pH 7.5, 130 mM NaCl, 500 μM $MgCl_2$, 50 μM $CaCl_2$.

In some cases the membranes were prepared by a freeze and thaw procedure: the cells were washed twice with TBS, resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 75 μg/ml phenylmethylsulfonyl fluoride, and 1 mM EDTA and disrupted by three cycles of freezing at −70 °C and thawing at 37 °C.

Determination of the Protein Stability by Pulse and Chase Experiments—48 h after infection the cells were washed twice, incubated with methionine-deficient minimal Eagle's medium (Invitrogen), and buffered to pH 6.2 with 25 mM MES-KOH. After 20 min at 27 °C, the same medium containing 200 μCi/ml [^{35}S]Met (Amersham Biosciences) was added and incubated for 3 h at 27 °C. A portion of the cells was collected (time = 0). To the remainder a 10,000 excess of cold Met in TNM-FH, 10% fetal calf serum, 50 μg/ml gentamicin was added, and the cells were transferred to 27 °C. Membranes were prepared from them by the freeze and thaw method described above, solubilized in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1% SDS and boiled for 5 min. Membrane proteins corresponding to 3 × 10^6 cpm were diluted in 500 μl of NEM (50 mM Tris-HCl, 7.5, 150 mM NaCl, 1 mM EDTA, 0.2% gelatin, 0.1% NaN$_3$, 0.2% SDS, 0.3% Nonidet P-40) and mixed with 1–2 μl of monoclonal antibody 5F10. After gentle shaking for 1–2 h at 4 °C, 20–30 μl of protein A-Sepharose CL-4B were added, and the incubation was continued for 2 h or overnight at 4 °C. The pellet was washed twice with NEM, twice with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% NaN$_3$, 0.3% Nonidet P-40, twice with 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, and resuspended in SDS-PAGE loading buffer.

Activity Measurements—The ATPase activity was measured as described in Ref. 37. The assays were performed on 10–30 μg of membrane proteins or, in the case of the purified protein, with 50–200 ng of protein in 500–1000 μl of 120 mM KCl, 30 mM HEPES-KOH, pH 7.2, 1 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM EDTA, 1 μM thapsigargin, and 5 μg/ml oligomycin. The amount of $CaCl_2$ needed to produce the free calcium concentration desired was calculated as described in Ref. 38. The formation of the phosphoenzyme intermediate from ATP was followed as described before (18): additional details are given in the legends for the figures. In brief, total membrane proteins or purified enzymes were diluted in 20 mM MOPS-KOH, pH 6.8, 100 mM KCl in the presence of different concentrations of Ca^{2+}, La^{3+}, or EGTA. The reaction was started by the addition of [γ-^{32}P]ATP (100–3000 Ci/mmol) on ice and stopped after 20 s by the addition of 6% trichloroacetic acid, 1 mM KH_2PO_4. The pelleted proteins were separated on acidic gels (39). After drying, the gels were exposed at −70 °C for 2–5 days.

SDS-PAGE, Western Blotting, and Protein Determination—Proteins were separated on SDS-polyacrylamide gel electrophoresis as indicated in Refs. 40 or 39. Protein concentrations were determined as described in Ref. 37. In some cases the gels were stained with a silver impregnation method (41). The proteins were transferred to nitrocellulose or to polyvinylidene difluoride membranes (Schleicher
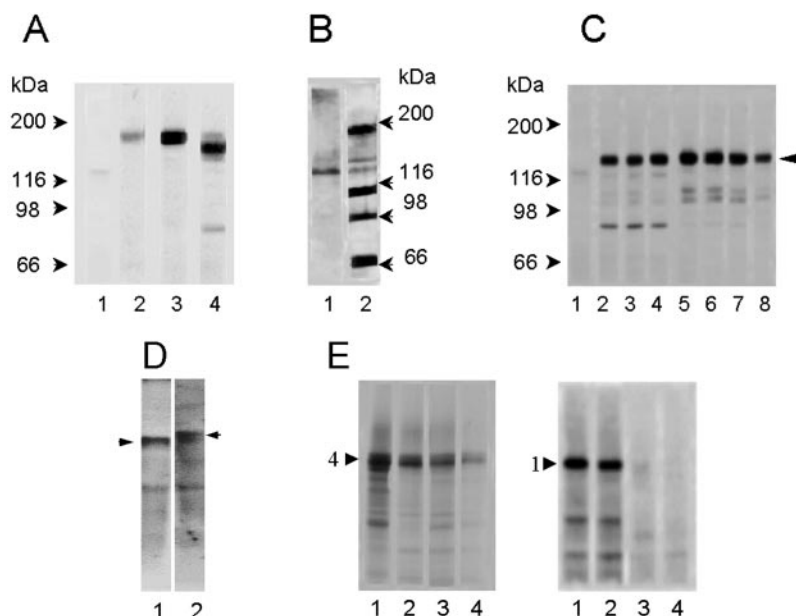
FIG. 1. **Expression, stability, and purification of the human PMCA1 pump expressed in Sf9 cells.** *A*, membrane proteins prepared from Sf9 cells (*lane 1*, 10 μg) or from Sf9 cells infected with PMCA1 (*lane 2*, 10 μg), PMCA2 (*lane 3*, 5 μg), and PMCA4 (*lane 4*, 5 μg) recombinant baculoviruses were transferred to nitrocellulose sheets and stained with the 2A polyclonal antibody (13). Similar results were obtained with the 5F10 antibody (56). Quantitative analysis of the bands indicated that the amount of expressed PMCA1 protein was 5- to 10-fold lower than that of the PMCA4 and PMCA2 isoforms. Densitometric quantification of the Western blotting in Fig. 3C yielded the following results, expressed in arbitrary units/μg membrane proteins: PMCA1 = 1.4, PMCA2 = 13, PMCA4 = 10.6. *B*, the PMCA1 protein was purified from Sf9 cells infected with the recombinant viruses by affinity chromatography on calmodulin-Sepharose. 50–100 ng of PMCA1 protein were separated by SDS-PAGE and visualized by silver staining (*lane 1*). The identity of the protein was confirmed by Western blotting (not shown). Protein markers are given in *lane 2*. Samples of purified PMCA4 and PMCA2 pumps yielded similar results (not shown). *C*, Western blotting of 20 μg of membrane proteins prepared from Sf9 cells (*lane 1*) or from Sf9 cells infected with seven different recombinant PMCA1 baculoviruses (*lanes 2–8*). The nitrocellulose sheet was incubated with the 5F10 monoclonal antibody. The molecular mass of the protein markers is given on the *left*. *D*, Northern blot of 20 μg of total RNA isolated for Sf9 infected with PMCA4 or PMCA1 recombinant baculoviruses. The blots were hybridized with digitoxigenin-labeled probes for PMCA4 (4) or PMCA1 (1). *E*, stability of the PMCA1 and PMCA4 proteins expressed in the Sf9 cells. Sf9 cells were infected with PMCA4 and PMCA1 recombinant baculoviruses, labeled for 3 h with [^{35}S]Met (*lane 1*) and chased with an excess of cold Met for 4 h (*lane 2*), 18 h (*lane 3*), and 25 h (*lane 4*). Membrane proteins were prepared by the freeze and thaw method, solubilized, and immunoprecipitated with the 5F10 antibody. The immunoprecipitated proteins were fractionated by SDS-PAGE gels. The dried gels were exposed at -70 °C for 3–5 days. The *arrows* on the *left* of the gels indicate the position of the pumps.

& Schuell) according to Ref. 42. The blots were processed essentially as described by Stauffer *et al.* (13) in the presence of PMCA-specific antibodies. After incubation with 1% bovine serum albumin in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20), the nitrocellulose was treated with monoclonal antibody 5F10 (1:2000 in TBST) or polyclonal antibodies 2A, 1N, 2N, and 4N (1:500 in 0.5% bovine serum albumin, TBST) for 1 h at room temperature. After a further 30-min incubation with alkaline phosphatase coupled to goat anti-mouse or goat anti-rabbit immunoglobulins (DAKO, Glostrup, Denmark), the immunocomplexes were detected with 66 μl of nitroblue tetrazolium and 33 μl of 5-bromo-4-chloro-3-indolyl-phosphate solutions (Promega) in 10 ml of alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 5 mM MgCl$_2$, and 1 mM CaCl$_2$).

Calmodulin Overlay—The proteins were transferred to nitrocellulose membrane sheets (42). Nonspecific binding was blocked by 1% bovine serum albumin in TBSM (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM MgCl$_2$, 0.2 mM CaCl$_2$, 0.05% Tween 20). For control experiments (unspecific binding), 1 mM EGTA was included in TBSM. The blots were incubated separately with different concentrations of biotinylated calmodulin (10^{-7} to 10^{-10} M) in TBSM for 1 h, washed twice in TBSM, and incubated for another hour with avidin-coupled alkaline phosphatase in TBSM. The filters were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate in 10 ml of alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 5 mM MgCl$_2$, and 1 mM CaCl$_2$). In all experiments the amount of the pump present was carefully calibrated by Western blotting performed in parallel. Only blots in which an identical amount of pump was present were evaluated.

Phosphorylation of PMCA by the cAMP-dependent Kinase—Prior to the purification the membranes were treated with alkaline phosphatase (3 units/μg protein) (Roche Applied Science) for 10 min at 37 °C. After addition of DNase (20 μg/ml) and of RNase (10 μg/ml) on ice, the different PMCA isoforms were purified on calmodulin-Sepharose. The proteins were phosphorylated in a final volume of 400 μl of 20 mM

Hepes-NaOH, pH 7.4, 130 mM NaCl, 0.05% Triton X-100, 0.5 mg/ml phosphatidylcholine, 10 mM MgCl$_2$, 2 mM EDTA, 1 mM EGTA, 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol), 1–5 μg of reconstituted protein kinase A (Sigma) for 30–60 min at 37 °C. The phosphorylated proteins were treated with 0.2 M hydroxylamine for 30 min at room temperature and separated by SDS-PAGE.

Calpain Digestion of the Ca^{2+}-ATPases—100 μg of membrane proteins obtained from infected Sf9 cells were incubated with 10 μl of calpain (1.5–2.0 μg) at 25 °C. The medium (0.2 ml final volume) contained 10 mM Tris-HCl, pH 7.5, and 200 μM CaCl$_2$. A zero time point was taken before adding calpain. Aliquots were withdrawn at the times indicated in the legends for the figures, and the reaction was stopped by adding SDS gel loading buffer (17 mM Tris-HCl, pH 8.0, 10% urea, 1.7% SDS, 2% dithiothreitol, 1.7 mM EDTA, and bromphenol blue).

Formation of the Phosphorylated Intermediate by the Pumps after Proteolysis with Calpain—25 μg of membrane proteins of Sf9 cells were incubated without or with calpain (0.37–0.5 μg) for 30 min in 50 μl of 20 mM MOPS-KOH, pH 6.8, 100 mM KCl, 100 μM Ca^{2+}, and 100 μM LaCl$_3$. The phosphorylation was started by adding 0.2 μM [γ-^{32}P]ATP (500 Ci/mmol). After 30 s on ice, the reaction was stopped with 600 μl of cold 7% trichloroacetic acid, 10 mM Na$_2$HPO$_4$. The precipitated proteins were divided into two aliquots (20 and 5 μg) and separated by acidic SDS-PAGE (39). The portion of the gel containing the 20-μg aliquots was stained with Coomassie Brilliant Blue, dried, and exposed overnight at -70 °C with an intensifying screen. The other portion of the gel was subjected to Western blot analysis using the 5F10 antibody.

Trypsin Digestion of Membrane Protein-expressing PMCA Isoforms—100 μg of Sf9 cell membrane proteins were resuspended in 200 μl of 20 mM Hepes-KOH, pH 8.0, 100 mM KCl. After taking the zero time aliquot, 2 μg of trypsin (Promega) were added to start the reaction. 40 μl of the digestion mixture were withdrawn at different times, transferred to a tube containing 2 μl of soybean trypsin inhibitor (1 mg/ml), and separated by SDS-PAGE.
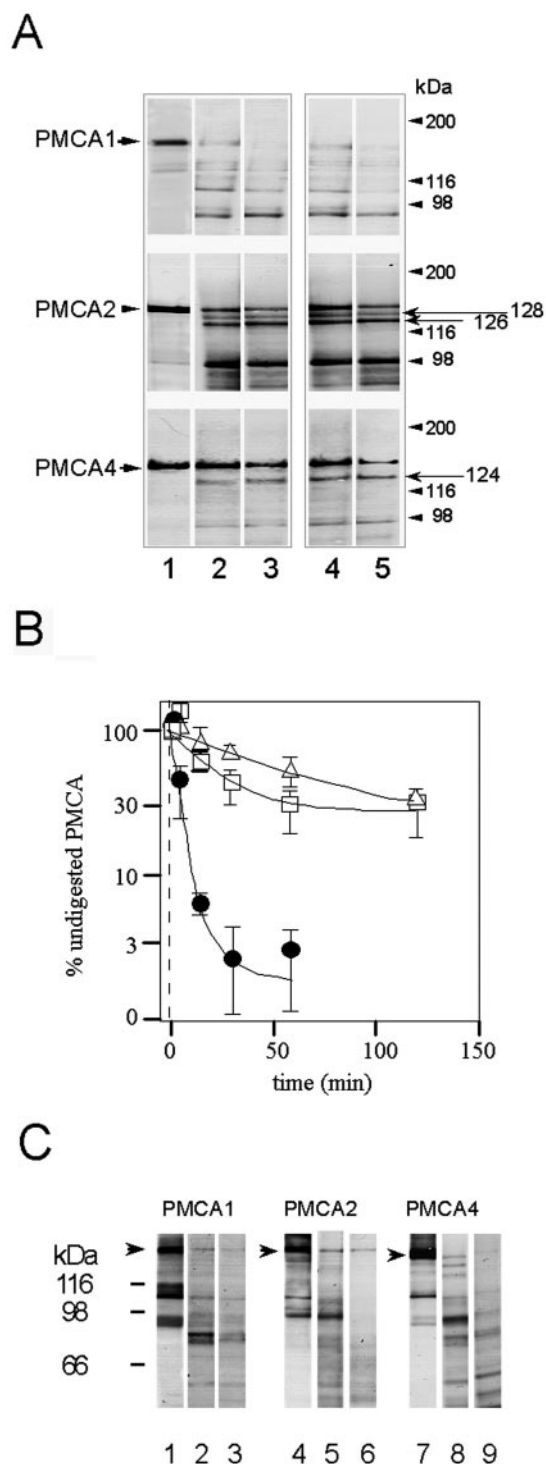
Isoform 1 of the PMCA Pump

FIG. 2. **Digestion of PMCA pump isoforms by calpain and trypsin.** *A*, digestion of PMCA1, PMCA2, and PMCA4 proteins by calpain. Membrane proteins from Sf9 cells expressing the PMCA1, PMCA2, or PMCA4 pumps were incubated with calpain as described under "Experimental Procedures" in the absence (*lanes 1–3*) or in the presence of 580 nM calmodulin (*lanes 4 and 5*). The digestion was stopped at 0 min (prior to the addition of the protease, *lane 1*), at 15 min (*lanes 2 and 4*), and at 60 min (*lanes 3 and 5*). 15 μg (in the case of PMCA1) or 1–2 μg (in the case of PMCA2 and PMCA4) of proteins were separated on SDS-PAGE, transferred to nitrocellulose, and incubated with the 5F10 antibody. *B*, densitographic quantification of the results of experiments similar to those of Fig. 2*A*. The 100% value on the ordinate refers to the amount of pump protein present before the addition of calpain. Only the amount of the full-length protein was considered in this analysis. The values are the average of two to five independent experiments. The *error bar* represents the S.D. *PMCA1*, ●; *PMCA2*, □; *PMCA4*, Δ. *C*, digestion of the PMCA1, PMCA2, and PMCA4 proteins by trypsin. 15

RESULTS

Characterization of the PMCA1 Pump Overexpressed in Sf9 Cells—Blots of membrane proteins obtained from Sf9 cells infected with recombinant baculoviruses for PMCA1, and with recombinant viruses for PMCA4 (15) and PMCA2 (18) used for comparison, were exposed to polyclonal antibody 2A (13), which recognizes all three isoforms. A reacting band of the expected size (135–140 kDa) was observed for the three proteins (Fig. 1*A*). Quantitative analysis showed that the expression level of PMCA1 was about 10 times lower than that of the other two isoforms. Despite the low level of expression, the PMCA1 protein could still be purified to homogeneity using a calmodulin-affinity column (Fig. 1*B*).

To establish whether the low level of expression of the PMCA1 pump was a property of the selected recombinant virus, the expression by seven independent isolated viruses was analyzed. All seven viruses directed the expression of a protein of about 140 kDa (Fig. 1*C*, *lanes 2–8*) that was recognized by the PMCA-specific antibody and that was absent in non-infected Sf9 cells (Fig. 1*C*, *lane 1*). All seven viruses expressed similar amounts of PMCA1 protein, and additional clones produced essentially the same results (not shown). Protein bands of lower molecular mass, which are likely to be degradation products of the pump, were also detected in the blots of all clones (Fig. 1, *C* and *E*). From some of the clones, viral DNA was prepared and analyzed by Southern blotting, yielding the expected band pattern (not shown).

To test whether the stability of the mRNA was responsible for the different expression level of the two isoforms, RNA was prepared from Sf9 cells infected with recombinant PMCA1 and PMCA4 baculoviruses and characterized by Northern blotting using specific cDNA probes (Fig. 1*D*). Signals of similar intensity were obtained for the mRNAs of the two pumps, showing that the differences in the level of expression were not explained by a transcription difference. The stability of the expressed proteins was therefore tested in pulse and chase experiments (Fig. 1*E*). 18 h after labeling the Sf9 cells with][35S]Met and chasing them with cold Met, only trace amounts of PMCA1 (less than 5–10% of the original amount) were detected (Fig. 1*E*, *lane 3*), whereas a significantly higher amount of PMCA4 was still present. Thus, the lower level of expression of PMCA1 was apparently due to the peculiarly low stability of the protein in Sf9 cells.

Proteolysis of the Expressed Pump Isoforms—To explore the reasons for the low stability of the PMCA1 pump, membranes of Sf9 cells expressing pump isoforms PMCA1, PMCA2, and PMCA4 were incubated with trypsin or with calpain (Fig. 2). The degradation pattern and the kinetics of the digestion of the three isoforms by calpain were different: *i.e.* the PMCA1 protein was digested much faster (Fig. 2, *A* and *B*) and failed to generate significant amounts of long-lasting products larger than 100 kDa, even when the reaction was stopped after only 2 min (not shown). By contrast, in agreement with previous results on the purified pump of erythrocytes (30), calpain digestion of the overexpressed PMCA4 protein (Fig. 2*A*) produced a relatively stable major polypeptide of 124 kDa. Calpain digestion of the PMCA2 protein resulted in the appearance of two polypeptides of 126–128 kDa (Fig. 2*A*) and of additional fragments of lower molecular mass (Fig. 2*A*). Addition of calmodu-

μg of membrane proteins from Sf9 cells expressing the PMCA1 pump (*lanes 1–3*) and 1–2 μg of membrane proteins from Sf9 cells expressing the PMCA2 (*lanes 4–6*) or PMCA4 pumps (*lanes 7–9*) were incubated with trypsin (protein/trypsin weight ratio, 50:1) for 0.1 min (*lanes 1, 4,* and *7*), 1 min (*lanes 2, 5,* and *8*), and 10 min (*lanes 3, 6,* and *9*). The samples were processed as described above for the digestion with calpain.
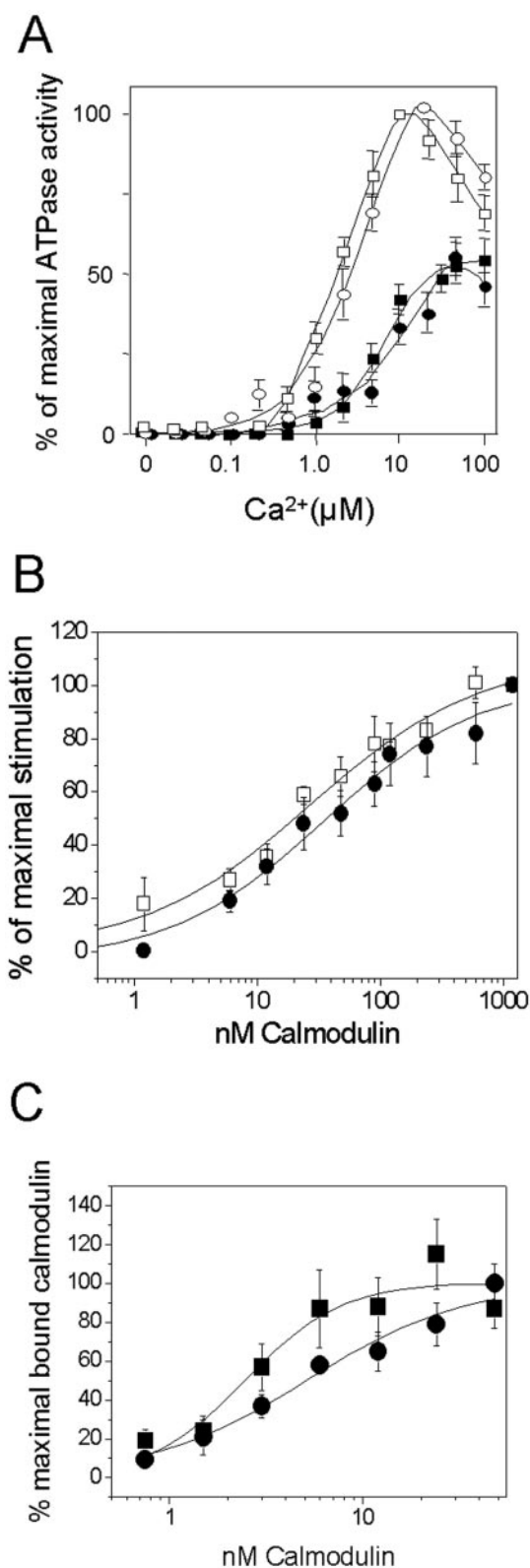
FIG. 3. **Biochemical properties of the PMCA1 pump protein expressed in Sf9 cells.** *A*, Ca^{2+} dependence of the ATPase activity of the purified pumps. The ATPase of the PMCA1 ($\square$,■) and PMCA4 ($\bigcirc$,●) proteins was determined in the absence (●,■) and in the presence ($\square$,$\bigcirc$) of 1.5 μM calmodulin. Each value represents the average of three independent experiments. *B*, calmodulin dependence of the Ca^{2+} ATPase activity of the PMCA1 ($\square$) and the PMCA4 pumps (●) in the presence of 5 μM free Ca^{2+}. The percent stimulation was calculated according to the following formula: (A−AB)/(Amax−AB), where A was the activity at a given concentration of calmodulin, Amax corresponded to the maximal activity at saturating concentrations of calmodulin, and AB represented the activity in the absence of calmodulin. The data are the

lin to the membranes in a concentration known to maximally stimulate the overexpressed pump (15, 18) (see Fig. 3*A*) failed to influence the proteolytic cleavage pattern of the three isoforms (Fig. 2*A*, *lanes 4* and *5*). At variance with calpain, the digestion pattern of the three isoforms with trypsin was more similar (Fig. 2*C*); even if the proteolytic process produced different polypeptides, all isoforms accumulated products in the 70–80-kDa molecular mass range. Fragments of this size had already been observed in previous trypsin experiments on the purified erythrocyte pump (43).

Biochemical Properties of the PMCA1 Pump Protein Expressed in the Sf9 Cells—The low expression level of PMCA1 made the direct measurement of the Ca^{2+} dependence of the ATPase activity in the membranes of Sf9 cells difficult. It was therefore decided to purify the expressed protein by calmodulin affinity chromatography on detergent extracts of Sf9 cell membranes (Fig. 1*B*, *lane 1*). The ATPase activity of the purified PMCA1 protein had the same Ca^{2+} dependence of the PMCA4 pump prepared under the same conditions (Fig. 3*A*). The two isoforms had equivalent V_{max} and K_m responses to the increase of added Ca^{2+} in the presence of calmodulin (Fig. 3*B*). The calmodulin dependence of the ATPase activity of PMCA1 was compared with that of PMCA4 and was found to be essentially the same (Fig. 3*B*). The binding of calmodulin was also estimated in gel overlay experiments in which the affinity of PMCA1 for calmodulin appeared to be slightly higher than that of PMCA4 (Fig. 3*C*).

A strong radioactive band of about 140 kDa was observed in extracts of membrane proteins of Sf9 cells infected with PMCA1 baculoviruses after incubation with [γ-^{32}P]ATP in the presence of La^{3+} and Ca^{2+} (Fig. 4*A*). These are conditions that promote the formation of the phosphoenzyme intermediate of the PMCA pump while inhibiting the formation of that of the sarcoplasmic/endoplasmic reticulum (SERCA) pump. No radioactive band was observed in control Sf9 cells (Fig. 4*A*, *lane 2*) or when 1 mM EDTA was included in the reaction medium (Fig. 4*A*, *lanes 1* and *3*). The radioactive band was sensitive to NH_2OH, and its intensity was strongly decreased by removing La^{3+} from the Ca^{2+}-containing medium, as shown for the PMCA4 (Fig. 4*A*, *lanes 5–7*). Similar results were obtained with PMCA2 (not shown, but see Ref. 18). A band of about 110 kDa was also observed in the samples incubated with Ca^{2+} and La^{3+}. This band was the phosphorylated intermediate of the endogenous SERCA pump, because the intensity of this band was strongly reduced when the membranes were preincubated with thapsigargin (see also Ref. 44). Another band at 96 kDa, which was present in EGTA and strongly inhibited by La^{3+}, was likely to correspond to the α-subunit of the Na^+/K^+ pump.

The formation of the phosphoenzyme intermediate by PMCA1 was studied in the presence of different concentrations of ATP. The PMCA1 pump was more efficient in the formation of the intermediate at low concentrations of ATP than the PMCA4 pump (Fig. 4*B*). The expressed PMCA1 (Fig. 4*B*) had an apparent K_m ATP of 0.053 μM, as compared with one of 0.34 μM estimated for PMCA4. PMCA2 and PMCA4 had been previously shown to have significantly different affinities for ATP (18). The affinity of PMCA1 was thus even higher than that of PMCA2 (the apparently K_m of the latter 0.2 μM) (18).

average of three to four experiments. An EC_{50} (calmodulin) for PMCA1 of 28 ± 8.6 nM and of 35 ± 11.7 nM for PMCA4 was determined by using a sigmoidal fitting. *C*, binding of calmodulin to the PMCA1 and PMCA4 pumps. The binding of biotinylated calmodulin was performed as described under "Experimental Procedures." The filters were quantified with the help of a scanner. (■), PMCA1; (●), PMCA4. Each value is the average of three independent experiments. The calculated EC_{50} values were 2.49 ± 0.41 nM for PMCA1 and 5.22 ± 0.58 nM for PMCA4.

Isoform 1 of the PMCA Pump

Previous work had shown that the cAMP-dependent kinase phosphorylates the PMCA pump isolated from erythrocytes, which is a mixture of isoforms 1 and 4 (45). A consensus site for the cAMP kinase (KRNSS) has been identified (31) in PMCA1 downstream of the calmodulin binding domain. Phosphorylation experiments using the cAMP-dependent kinase were thus performed on PMCA1, PMCA2, and PMCA4 (Fig. 4C). The results presented show that PMCA1 was phosphorylated to a much higher degree than the other isoforms, in which only very low levels of phosphorylation were obtained. Evidently, the consensus site, which is canonical in PMCA1, is less adequate in the other isoforms.

Characterization of the Proteolytic Products of the Pump Isoforms—To characterize functionally the fragments of the PMCA2 and PMCA4 isoforms generated after 30 min of cleavage by calpain, the fragments were separated by SDS-PAGE, blotted, and incubated with isoform-specific antibodies or with biotinylated calmodulin. In parallel, the formation of the phosphorylated intermediate was explored (Fig. 5). In the case of the PMCA4 protein, the fragment of a molecular mass of 124 kDa that was generated failed to react with biotinylated calmodulin and thus evidently lacked the calmodulin binding domain. The fragment was even more active in the phosphorylated intermediate assay than the original intact pump still present in the preparation (Fig. 5). This could possibly reflect previous findings that the removal of the C-terminal domain relieves the pump from autoinhibition, making it fully active (30, 46, 47) (see below). The pattern of digestion of the PMCA2 pump was more complex. Fragments of 130, 124, 95, and 42 kDa were generated; they evidently contained the N-terminal portion of the PMCA2 pump because they were recognized by the 2N polyclonal antibody (Fig. 5), which is specific for the N-terminal part of the PMCA2 protein (13). As expected, the 95- and the 42-kDa fragments were not recognized by calmodulin in the overlay experiments. However, a 45-kDa fragment that was recognized by the 5F10 monoclonal antibody but not by antibody 2N was also recognized by calmodulin (Fig. 5). Thus, it apparently contained the C-terminal portion of the pump, including the calmodulin binding domain. The large active fragments generated by the digestion of PMCA2 were active in the phosphorylated intermediate assay. It has so far proven impossible to identify conditions under which calpain could generate PMCA1 fragments active in the phosphorylation assay or which were recognized by calmodulin (not shown).

DISCUSSION

Cloning of the cDNA sequences has revealed numerous isoforms of the PMCAs (3). The cDNA for the PMCA1 was the first
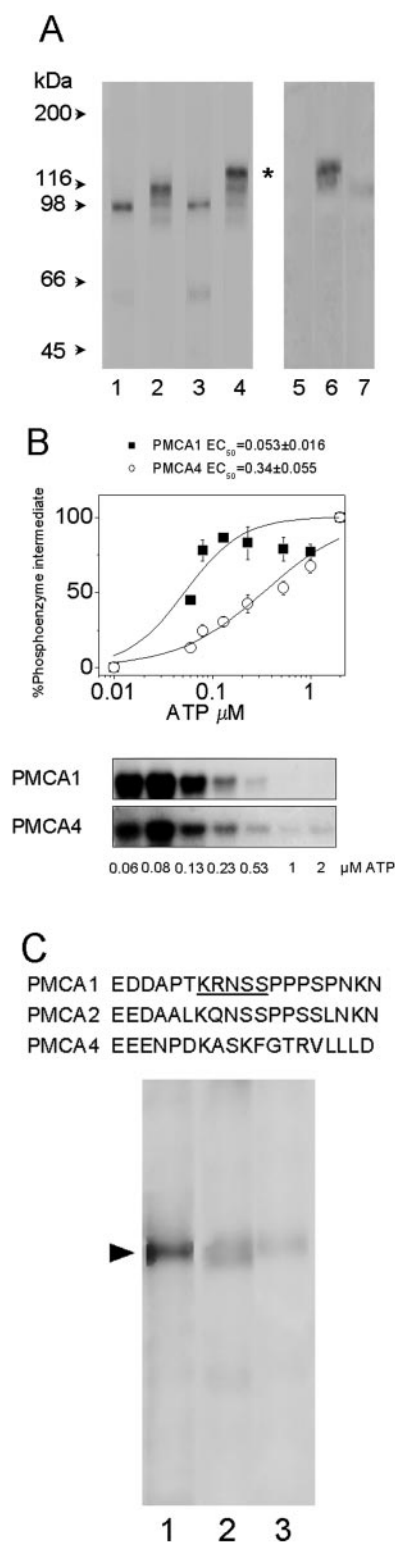
FIG. 4. **Formation of the phosphoenzyme intermediate and phosphorylation by cAMP kinase of the PMCA1 pump protein expressed in Sf9 cells.** *A*, formation of the phosphoenzyme intermediate from ATP. Membrane proteins obtained from Sf9 cells infected with a control virus (*lanes 1* and *2*, 30 μg) and with recombinant virus for PMCA1 (*lanes 3* and *4*, 30 μg) were phosphorylated with 0.4 μM [γ-^{32}P]ATP (250 Ci/mmol) in the presence of 1 mM EGTA (*lanes 1* and *3*) or in the presence of 200 μM CaCl$_2$ and 200 μM LaCl$_3$ (*lanes 2* and *4*) and processed as described under "Experimental Procedures." The PMCA4 expressed in Sf9 cells was purified as was done for PMCA1 (see Fig. 1*B*) and phosphorylated in the presence of EGTA (*lane 5*) or in the presence of 200 μM CaCl$_2$ and 200 μM LaCl$_3$ (*lanes 6* and *7*) followed by hydroxylamine treatment (*lane 7*). The dried gels were exposed at -70 °C for 2 days. The *asterisk* indicates the position of the PMCA phosphoen-

zyme. *B*, concentration dependence of the ATP effect on the formation of the phosphoenzyme. The reaction was performed in the presence of 200 μM CaCl$_2$, 200 μM LaCl$_3$, and different concentrations of ATP. In each experiment 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) were used and sufficient cold ATP was added to achieve the desired concentration. The radioactive bands corresponding to PMCA1 (■) and PMCA4 (○) were quantified by phosphorimaging. The data represent the average of three different experiments. A fitting using a sigmoidal equation was performed. The EC$_{50}$ values obtained are indicated *above* the graph. A typical experiment is shown at the *bottom* of the figure. The time of exposure of the autoradiogram was chosen so that the intensity of the phosphoenzyme intermediate of the two pumps had a similar intensity at 0.23 μM ATP. *C*, PMCA1 as a preferred substrate for the cAMP-dependent protein kinase. PMCA1 (100 ng, *lane 1*), PMCA4 (200 ng, *lane 2*), and PMCA2 (200 ng, *lane 3*) proteins purified on calmodulin columns were phosphorylated by the cAMP-dependent protein kinase. The reaction products were separated by SDS-PAGE and processed for autoradiography. The sequences around the consensus site for the cAMP-dependent protein kinase site in the three PMCA isoforms are shown at the *top*. The expected position for the PMCA pumps is indicated by the *arrow*.
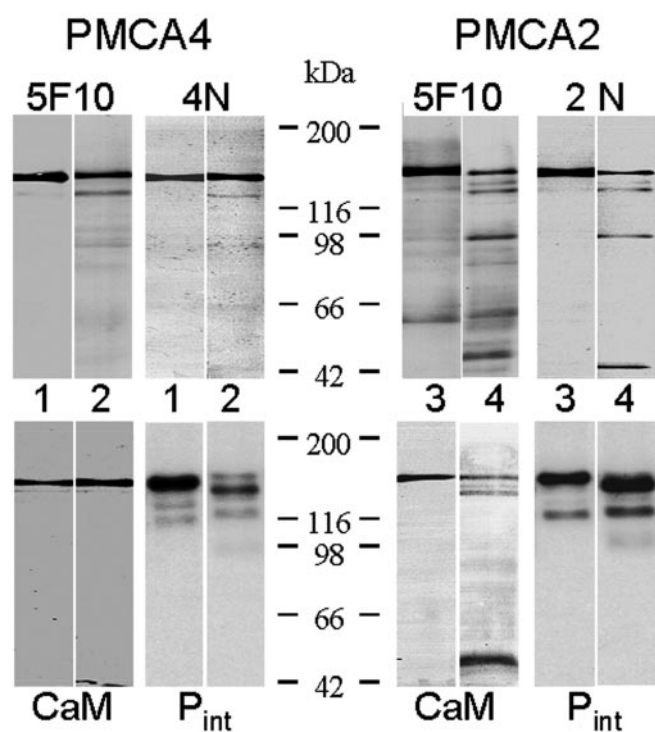
FIG. 5. **Products generated by the digestion of the PMCA2 and PMCA4 proteins with calpain.** Membrane proteins from Sf9 cells expressing the PMCA4 (*lanes 1* and *2*) and PMCA2 (*lanes 3* and *4*) pumps were digested with calpain for 0 min (*lanes 1* and *3*) or 30 min (*lanes 2* and *4*). 1–2 μg were separated by SDS-PAGE and transferred to nitrocellulose. The filters were incubated with the 5F10 monoclonal antibody (*5F10*), which is specific for the catalytic region of the pump (amino acids 724–783) (50), with the 4N antibody specific for the N-terminal domain of the PMCA4 pump, and with antibody 2N, which is specific for the N-terminal domain of the PMCA2 pump (13). A calmodulin gel overlay (using 2 μg of membrane proteins, see "Experimental Procedures") was performed with the same samples (*CaM*). Membrane proteins from Sf9 cells expressing the PMCA4 (*lanes 1* and *2*) and PMCA2 (*lanes 3* and *4*) pumps were incubated on ice with [γ^{32}P]ATP (P_{in}) as described under "Experimental Procedures" before (*lanes 1* and *3*) or after (*lanes 2* and *4*) 30 min of digestion with calpain. 20 μg of the phosphorylated protein samples were separated on an acidic gel and processed for autoradiography.

to be isolated (8, 32). Peptides encompassing the C-terminal region (48, 49), the N-terminal region (13), and the central region[2] have been expressed, but attempts to isolate or to construct full-length PMCA1 cDNA have been frustrated by DNA stability problems (23, 24). The only report describing the overexpression of an active PMCA1 isoform (23) refers to a terminally truncated variant termed PMCA1a, which corresponds to PMCA1CII in the nomenclature followed in this laboratory (1). The study did not characterize the biochemical properties of the pump nor did it compare them to those of other PMCA isoforms. Special efforts were thus made to produce a mutation-free full-length cDNA of the isoform (PMCA1CI or PMCAb in the alternative nomenclature) and to transfer the full-length coding sequence to a recombinant baculovirus expression vector. Because other PMCA isoforms had already been expressed in Sf9 cells, the use of this system has allowed the direct comparison of the biochemical properties of the isoforms.

The lower expression level of PMCA1 as compared with that of the PMCA4 and PMCA2 isoforms (15, 18) was unexpected. The recombinant viruses for PMCA1 behaved as those isolated for the PMCA2 and PMCA4 counterparts; thus, it was obvious

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[2] B. Seiz, unpublished observation.

to look for alternative explanations. In principle, the transcription efficiency of PMCA1 in Sf9 cells could have become inherently lower, for instance, because of the interference of the 5′-untranslated sequences with the transcription mediated by the polyhedrin promoter (34): At variance with PMCA4 and PMCA2 (15, 18, 24), in the case of PMCA1 the 5′-untranslated region was not entirely deleted. However, this was unlikely to be the reason for the lower protein yield, because the levels of mRNA for the pump isoforms in the Sf9 cells were practically identical. This also ruled out the instability of the PMCA1 mRNA as the reason for the lower expression. Finally, the PMCA1 protein could have been more sensitive to proteolytic degradation or, more generally, have a higher turnover than PMCA4 and PMCA2. This was indeed found to be the case, as revealed by the pulse and chase experiments of Fig. 1*E*. Because PMCA pumps and PMCA pump mutants expressed in Sf9 cells behave like those expressed in other cell lines (21), this is likely to reflect a peculiar property of the PMCA1 pump and could, for instance, also explain why so much more PMCA4 than PMCA1 protein has been detected in red blood cells, at variance with other cells (13); erythrocytes do not synthesize proteins, therefore, the more stable isoform PMCA4 is preferentially enriched with respect to PMCA1.

The sequence identity of the four basic isoforms of the plasma membrane Ca^{2+} ATPases averages 75% (51). It was thus surprising to find that the three isoforms studied (PMCA1, PMCA2, and PMCA4) had such a significant difference in the susceptibility to calpain. Because the sensitivity to trypsin was similar, the effect of calpain was evidently specific. It could thus be (cautiously) suggested that calpain controls the turnover of the pump isoforms, regulating their concentration. One could mention in this context that the erythrocyte calcium pump has been shown to be a preferred substrate of calpain *in vivo* (52). At variance with the results on the purified enzyme (30, 53) the degradation of the erythrocyte pump *in vivo* rapidly proceeded past the stage of the 124-kDa product until the pump disappeared completely. In light of the results presented here on the two overexpressed erythrocyte isoforms, PMCA4 and PMCA1, it appears likely that their complete disappearance *in vivo* was the result of the action of other (unknown) proteases attacking the (initial) products of calpain cleavage. At least in erythrocytes, then, the result of the activation of calpain would not be the increased ejection of calcium by the stimulated PMCA pump but its inhibition due to its complete degradation.

The calpain proteolytic pattern of the PMCA4 protein was similar (if not identical) to that of the isolated erythrocyte PMCA pump, *i.e.* a major polypeptide of 124 kDa was generated that lacked the calmodulin binding domain (30). The digestion of the PMCA2 protein by calpain produced instead a more complex pattern. The two high molecular mass fragments (130 and 124 kDa) contained the N-terminal part of the protein and still bound calmodulin, but another calmodulin binding peptide of about 45 kDa was also generated. Evidently, calpain attacked different sites in the PMCA4 and PMCA2 proteins. The finding was somewhat unexpected, because two of the major calpain cleavage sites in the PMCA4 protein (Arg-1089 ↓ Arg-1090; Asn-1099 ↓ Arg-1100; Ref. 30) are conserved in the sequences of the PMCA2 pump (51).

The basal activity of the purified erythrocyte PMCA pump is stimulated by calpain, because of the removal of the autoinhibitory calmodulin binding domain (30). The activation has been detected in both the PMCA4 and the PMCA2 proteins, even if the work presented here has shown that the cleavage by calpain interests different sites. This suggests that the removal of the C-terminal portion of the pump may weaken the interaction of the autoinhibitory calmodulin binding domain with its

receptor sequence next to the active site of the pump. Alternatively, it could be suggested that an inhibitory sequence acts downstream the calmodulin binding domain (54, 55).

Although the PMCA1 pump was expressed at a lower level than the PMCA4 and PMCA2 isoforms, it still proved possible to purify low amounts of it and to establish that its Ca^{2+} and calmodulin-dependent ATPase activity did not differ from that of PMCA4. However, the overlay experiments with biotinylated calmodulin revealed a small but reproducible difference in the affinities of the two pumps for the activator. The higher affinity of PMCA1 could, in principle, be related to the stronger affinity of its autoinhibitory calmodulin binding domain for the catalytic region of the pump (46, 47). However, the difference in K_m was too small (2.5 *versus* 5.2 nM) to result in a measurable difference when the stimulation of the ATPase activity by calmodulin was measured. Surprisingly, the PMCA1 and PMCA2 enzymes showed a much higher affinity for ATP in the experiments on the phosphoenzyme formation from ATP than PMCA4. The difference was even more pronounced for PMCA1 than for PMCA2 (18), but it would be difficult to suggest that it would be significant under physiological conditions, where the ATP concentration is much higher than in the experiments reported here and in previous work (18). Nevertheless, it indicates differences in the kinetic (mechanistic) properties of the pumps. Another interesting point concerns the phosphorylation by the cAMP-dependent kinase. In agreement with earlier observations on the erythrocyte enzyme (45, 31), the work has shown that PMCA1 is a better substrate for protein kinase A than isoforms 2 and 4. It remains to be established whether the limited phosphorylation of the other isoforms is functionally significant, *i.e.* whether it influences the activity of the pump *in vivo*.

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Expression, Purification, and Characterization of Isoform 1 of the Plasma Membrane Ca $^{2+}$ Pump: FOCUS ON CALPAIN SENSITIVITY

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